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14. ABSTRACT: Data from our lab and others indicate that <u>normal breast</u> cells do not express the erythropoietin receptor (EpoR); conversely, <u>breast cancer</u> cells express functional EpoR. Expression of EpoR appears greatest in poorly oxygenated tumor regions and in patients with negative estrogen receptor status, a sign of more aggressive disease. In addition one study demonstrated that the EpoR gene is overexpressed in patients with micrometastatic disease. The differential expression of EpoR between normal and cancerous breast cells has led us to hypothesize that <u>acquisition of EpoR expression by mammary epithelial cells may be part of malignant transformation and may impact disease progression and metastasis</u> . Our proposal investigates changes in mammary epithelial cell biology associated with acquisition of EpoR expression. Our results will lead to a better understanding of the appropriate use of Epo in breast cancer patients and may lead to development of the EpoR as a novel therapeutic target in the treatment of breast cancer.					
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Introduction

Normal mammary epithelial cells/cell lines do not express the erythropoietin receptor (EpoR), while breast cancer cells (and cell lines) are EpoR-positive (1-3). Neither the functional significance (4-6) of EpoR on these nonhematopoietic cells nor that of their differential expression on normal vs. malignant mammary epithelial cells is understood. Erythropoietin (Epo) has widespread clinical application in the treatment of breast cancer (CaBr), where it has been demonstrated to relieve disease- or treatment-related anemia and fatigue, to improve cognitive function, and to decrease tumor/tissue hypoxia. However, several recent clinical trials have reported (7-9) that Epo treatment of at least some cancer patients (including CaBr patients) may be associated with decreased overall survival. Taken together, the data have led us to hypothesize that EpoR acquisition 1) may be part of the process or malignant transformation for mammary epithelial cells, and 2) the EpoR may be functioning like an oncogene for mammary epithelial cells. Our working hypothesis in the current grant is that **acquisition** of EpoR by mammary epithelial cells may influence the oncogenic process and that Epo may be a growth and survival factor for CaBr cells. To test our hypothesis our original grant application proposed to insert the human EpoR into human mammary epithelial cells and, conversely, to downregulate the EpoR in human CaBr cells. The effect of EpoR acquisition (or loss) is to be assessed by a series of gain- or loss-of-function studies.

Body

Verification of EpoR status of human mammary epithelial and breast cancer cells. We started our investigation by screening multiple human breast cancer (CaBr) cell lines, available in our laboratory, for the presence of EpoR. The cell lines that we selected varied in their *in vitro* and *in vivo* growth characteristics, as enumerated below (**Table 1**).

Table 1. Characteristics of breast cancer cell lines used in this study.

Cell line	Tumorigenicity <i>in vivo</i>	Invasiveness <i>in vitro</i>	Phenotype/ genotype	Morphology on plastic	ER/PgR	other
MCF-7	poorly (estrogen dependent)	weakly	luminal epithelial-like	polygonal	+/+	typical epithelial markers
T-47D	poorly (estrogen dependent)	weakly	luminal epithelial-like	polygonal	+/+	typical epithelial markers
SK-BR-3	yes	weakly	weakly luminal epithelial-like	clusters of weakly attached cells	-/-	amplified <i>ERBB2</i>
MDA-MB-231	highly	highly	stromal/mesenchemal	fibroblastoid	-/-	over-express vimentin
Hs578T	yes (semisolid medium)	highly	stromal/mesenchemal	fibroblastoid	-/-	over-express vimentin

We confirmed, by RT-PCR, that all of the CaBr lines we had selected express the gene for the human EpoR (**Figure 1**). Based upon the RT-PCR results, we concluded, therefore, that MCF7 (lane 3) would be a representative line to use as our EpoR-positive CaBr cell line. We further confirmed that the "normal" (nontumorigenic) mammary epithelial cell line, MCF10A (lane 4), did NOT express EpoR and would, therefore, be suitable as our EpoR-negative mammary epithelial cell line.

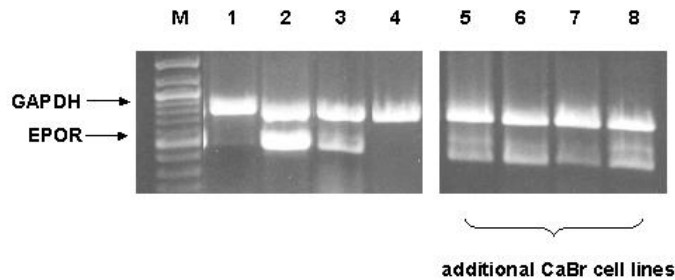


Figure 1: RT-PCR amplification of a 485 bp fragment of the human EpoR gene from human breast cancer cell lines. RNA was prepared from all cells using TriZol reagent, according to manufacturer' methods and used for subsequent RT-PCR co-amplification of EpoR and GAPDH fragments. lane 1: BaF3 pro-B cells, which lack EpoR (negative control); lane 2: BaF3/EpoR cells, transfected with the full-length human EpoR gene (positive control); lane 3: **MCF7** (EpoR positive) CaBr cell line; lane 4: **MCF10A** (EpoR negative) mammary epithelial cells; lanes 5-8: additional (EpoR positive) CaBR cell lines – SKBR, MDA-MB231, Hs578T, and T47D, respectively.

Downregulation of EpoR in human breast cancer cells, using antisense technology. As proposed in the original grant application, we used pcDNA3.1/EpoR AS (pcDNA3.1^(c) vector, containing the full length human EpoR cDNA inserted in the "antisense orientation" - i.e. the orientation that would result in transcription on EpoR "antisense message"), to transfect MCF7 cells. DOTAP (Roche) lipid-mediated transfection was utilized. Control cells were transfected with empty pcDNA3.1^(c) vector. This method for downregulation of gene expression using full-length "antisense cDNA" was selected because of our previous success with the method (and with the particular construct) (10). Following G418 selection and growth of G418 resistant cells, single-cell cloning resulted in the growth of numerous clones. Forty-eight robust clones were selected for further growth and expansion. These cells have been designated MCF7/EpoR↓, to designate cells with downregulated EpoR.

Two-step RT-PCR, with gene-specific primers, was utilized to determine 1) how many of the selected MCF7/EpoR↓ clones contained the full-length AS EpoR sequence and 2) how many of the MCF7/EpoR↓ clones that were successfully transfected with the AS construct had decreased levels of EpoR expression. Our survey showed that 41 of the 48 clones contained the full-length AS construct.

A problem that we have encountered with our selected strategy of using the full-length EpoR antisense cDNA construct is that since the AS and sense strands are of the same length, it has been extremely difficult to determine, by RT-PCR, whether downregulation of EpoR (sense strand) expression has occurred. We have been able to demonstrate the *presence* of AS in the cells, by selecting primers that encompass [portions of] the vector sequence as well as the EpoR coding sequence. However, we have not been able to devise a primer set that would be specific for the sense strand (i.e. the actual cDNA for sense and antisense constructs has the identical sequence – only their orientation is reversed) that would allow strand-specific amplification. We have made two attempts at real-time and semi-quantitative RT-PCR to detect strand specific message (i.e. downregulation of EpoR expression) without success. Because MCF7 cells are not Epo-dependent (although they are Epo-responsive – *see below*) there is some subjectivity in

assessing whether and/or to what degree we may have downregulated EpoR expression in our clones.

In discussion with colleagues in the laboratory we have come up with two possible solutions to this problem of positively identifying EpoR-downregulated clones. The first employs an EpoR AS construct that is "tagged", so it would not amplify with primers from sense sequence. The second involves the use of si/sh RNA, rather than a full-length AS sequence, for EpoR gene downregulation. The design and testing of candidate siRNAs is currently underway by colleagues in the laboratory.

Insertion of the full-length EpoR gene into human mammary epithelial cells. As proposed in the original grant application, we used pcDNA3.1/EpoR (pcDNA3.1⁽⁺⁾ vector, containing the full length human EpoR cDNA as insert) to transfect (EpoR negative) MCF10A cells with the human EpoR gene. As with the antisense cDNA transfection discussed above, DOTAP lipid-mediated transfection was used for each of the three separate transfections we performed. Control cells were transfected with empty pcDNA3.1⁽⁺⁾ vector. 600 ug G418/ml was applied as a selection reagent.

We have grown and expanded the G418-resistant cells (designated as MCF10A/EpoR). A first attempt at single-cell cloning of MCF10A/EpoR yielded nearly 100 positive wells but eventually failed due to an unfortunate incident of fungal contamination of the entire tissue culture incubator. A second single-cell cloning is in progress.

We utilized one-step RT-PCR, with gene-specific primers, to amplify a 485-bp fragment of the EpoR gene from MCF10A/EpoR cells derived from two independent transfectants (**Figure 2**). This demonstrates that we were successful in introducing the EpoR gene into MCF10A cells.

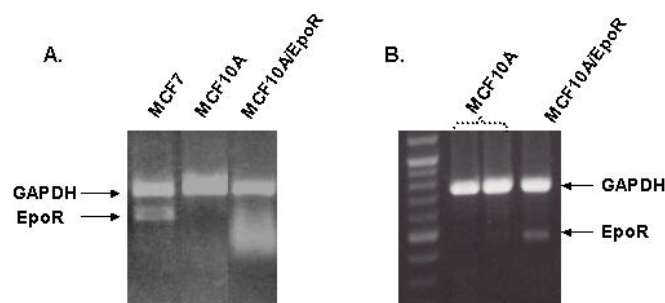


Figure 2: RNA was prepared from (EpoR+) MCF7 cells, from (EpoR-) MCF10A cells, and from two independently-generated preparations of MCF10A/EpoR cells (using TriZol reagent and following manufacturer's recommendations). EpoR -specific primers were used to amplify a 485 bp fragment of the human EpoR gene (a 797 bp fragment of the GAPDH gene was co-amplified as loading control and as a measure of RNA integrity). Panel A (left): The EpoR gene fragment is amplified from MCF7 cells (left lane) and from MCF10A/EpoR (right lane), but not from MCF10A cells (middle lane). Panel B (right): A 485 bp EpoR gene fragment is amplified from the MCF10A/EpoR transfectants (right lane), but not from either of two samples of MCF10A cells (middle two lanes).

Investigation of changes in behavior of mammary epithelial cells upon acquisition of EpoR expression. To reiterate, our hypothesis is that acquisition of EpoR expression by mammary epithelial cells may be related in some way to the oncogenic process in CaBr. Therefore,

following transfection of MCF10A cells with the EpoR (MCF10A/EpoR cells), we have begun preliminary experiments designed to test the effect of EpoR acquisition on the *in vitro* biology of the cells. These are preliminary experiments, and optimal conditions are still being worked out. In addition, we will repeat all of the experiments once we have clonal populations of MCF10A/EpoR cells.

One of our initial observations is that MCF10A/EpoR cells appear to grow in culture more rapidly than do the "parental" MCF10A cells (**Figure 3**). Of course, this "qualitative" observation needs to be followed up with quantitative growth curves for the two cell types, and this work is ongoing.

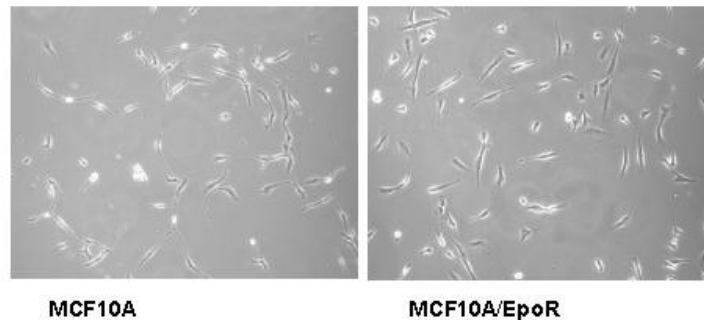


Figure 3: Equal numbers of (parental) MCF10A [left] and (transfected) MCF10A/EpoR [right] were plated in standard growth medium. Culture dishes were observed after 48 hr. The qualitative observation from this initial experiment is that the growth rate of MCF10A/EpoR is faster than that of the parental MCF10A cells.

We also wanted to determine whether acquisition of EpoR affected colony formation in soft agar. It has been established that MCF7 cells form colonies in soft agar, and our initial experiment, shown below, suggests that Epo stimulates the growth of MCF7 soft agar colonies, by increasing the number of colonies formed (**Figure 4**). This is, to our knowledge, the first demonstration of the effect of Epo on MCF7 colony formation. MCF10A cells do not form colonies in soft agar, and our results confirm this. In the present experiment, at least at the 1-week time point, it is not definitive whether MCF10A/EpoR cells form colonies in soft agar (note: there are some very small colonies starting to appear in the MCF10A/EpoR wells, but these do not visualize well in the photographs, and we will need to determine whether these persist as "real" colonies). These cultures will be examined for up to 6 weeks and, in addition, we will be working to optimize the soft agar culture conditions to see if this affects our preliminary observation.

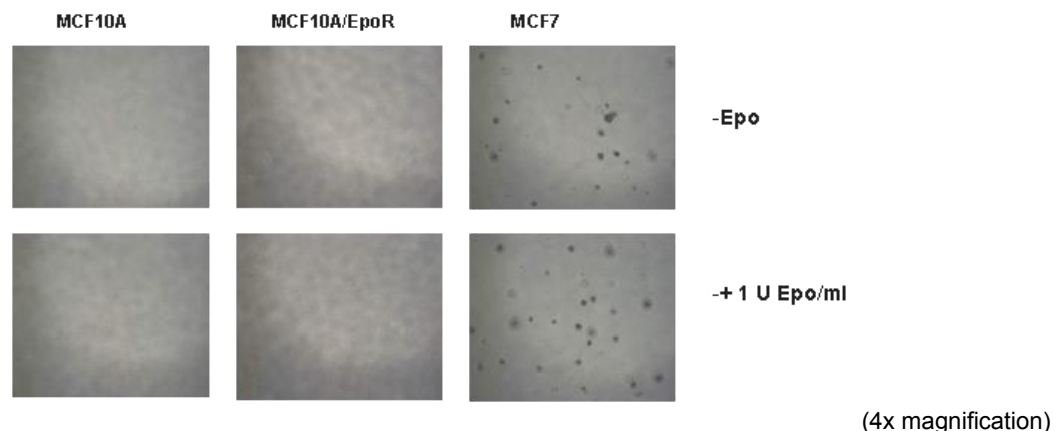


Figure 4: Growth of MCF10A (left column), MCF10A/EpoR (middle column) and MCF10A/EpoR (right column) in soft agar culture. 2500 cells were plated per well, in 12-well plates, in RPMI – 5% FBS – 0.35% agarose. Cells were plated in the absence (top row) or presence of 1 U rhEpo/ml (bottom row). Cultures were photographed after 1-week incubation at 37°, 5% CO₂.

Finally, we have generated "chronically Epo-treated" MCF10A/EpoR cells, by passing aliquots of cells in the presence of 2U rhEpo/ml for 2 months. These cells - designated MCF10A/EpoR^{+2U^{rhEpo}/ml} - are now ready to be tested, along side MCF10A/EpoR cells, to determine how chronic Epo treatment affects their growth and survival.

Key Research Accomplishments

The following points summarize the accomplishments to date from this grant:

- We have introduced the human EpoR gene into EpoR-negative MCF10A mammary epithelial cells
- We have provided an initial demonstration that acquisition of EpoR gene expression by MCF10A cells (=MCF10A/EpoR cells) results in faster cell growth in culture, even in the absence of exogenous erythropoietin
- We have used antisense technology to downregulate EpoR gene expression in MCF7 cells (=MCF7/EpoR↓ cells).
- We have provided what we believe to be the first reported evidence that Epo augments the growth of MCF7 colonies in soft agar culture.

Reportable results

The following are the direct result of research funded by this grant:

- We have established 41 initial clonal lines of MCF7 breast cancer cells with downregulated EpoR (=MCF7/EpoR↓).

- We have established of two independent derivatives of MCF10A mammary carcinoma cells expressing the human EpoR (=MCF10A/EpoR) – establishment of clonal cell populations is in progress.
- We have established subpopulations of MCF10A/EpoR cells that have been chronically Epo-treated (=MCF10A/EpoR^{+2U Epo/ml}) and have begun to compare the Epo-treated and untreated cells in culture.
- We have applied for continued research funding (two grants) based upon this work and upon our working hypothesis that the EpoR may function as an oncogene in breast cells.
- We are preparing an abstract for submission to the American Association for Cancer Research annual meeting.

Conclusion

We successfully have introduced the EpoR gene into (nontumorigenic, EpoR negative) MCF10A cells and have begun gain-of-function studies aimed at determining how EpoR acquisition affects the in vitro biology of MCF10A/EpoR cells. We are continuing and expanding these studies aimed at determining the effect of EpoR acquisition on the cells.

We also have used "antisense technology" to downregulate EpoR expression in MCF7 CaBr cells. We have been hampered by technical problems in *detecting* downregulation of EpoR gene expression, but we have begun to address these problems by designing additional strategies for receptor downregulation in our system.

The reagents that we have prepared and now have at our disposal – MCF10A cells, MCF10A/EpoR, MCF10A/EpoR^{+2U Epo/ml}, MCF7, MCF7^{+2U Epo/ml}, and MCF7/EpoR↓ - are powerful tools that will allow us to carry out further studies designed to investigate the role of EpoR (acquisition) in mammary carcinogenesis.

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Appendix (CV for Laurie Feldman, PhD)**BIOGRAPHICAL SKETCH**

NAME		POSITION TITLE	
Laurie Feldman, Ph.D.		Assistant Professor of Medicine (Biochemistry)	
EDUCATION/TRAINING <i>(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)</i>			
INSTITUTION AND LOCATION	DEGREE <i>(if applicable)</i>	YEAR(s)	FIELD OF STUDY
Mount Holyoke College, South Hadley, MA	A.B.	1969-1973	biochemistry
Tufts University School of Medicine, Boston, MA	Ph.D.	1973-1980	biochemistry

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past

3 years and representative earlier publications pertinent to this application. PAGE LIMITATIONS APPLY. DO NOT EXCEED FOUR PAGES FOR THE ENTIRE BIOGRAPHICAL SKETCH PER INVESTIGATOR.

Research and Professional Experience

1981-1983	Postdoctoral Fellow, Department of Microbiology, Boston University School of Medicine, Boston, MA
1983-1986	Research Associate, Department of Biomedical Research, St. Elizabeth's Hospital, Boston, MA
1986-1988	Assistant Investigator, Department of Biomedical Research, St. Elizabeth's Hospital, Boston, MA
1986-1988	Assistant Professor, Department of Medicine, Tufts University School of Medicine, Boston, MA
1988-1990	Instructor in Medicine (Biochemistry), Harvard Medical School, Boston, MA
1988-1996	Principal Investigator, Division of Hematology and Oncology, Department of Medicine, New England Deaconess Hospital, Boston, MA
1991-	Assistant Professor of Medicine (Biochemistry), Harvard Medical School, Boston, MA
1996-	Principal Investigator, Division of Hematology and Oncology, Department of Medicine, Beth Israel Deaconess Medical Center, Boston, MA
1998-	Member, Beth Israel Deaconess Cancer Center, Boston, MA
2003-	Member, Dana Farber/Harvard Cancer Center, Boston, MA
2004-	FDA Oncology Drug Advisory Committee, consultant

Honors and Awards

1997	Focused Giving Award, Robert Wood Johnson Foundation
1995, 2002	Aid for Cancer Research Equipment Awards

1973 AB, Magna Cum Laude, Mt. Holyoke College
 1972 Louisa Stone Stevenson Prize in Chemistry, Mt. Holyoke College
 1970 Bernice Maclean Shapiro Prize in Biology, Mt. Holyoke College

Representative Research Publications

1. Feldman L, Stollar BD. Serological analysis of the H3-H4 histone complex. *Biochem.* 1977; 16:2767.
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